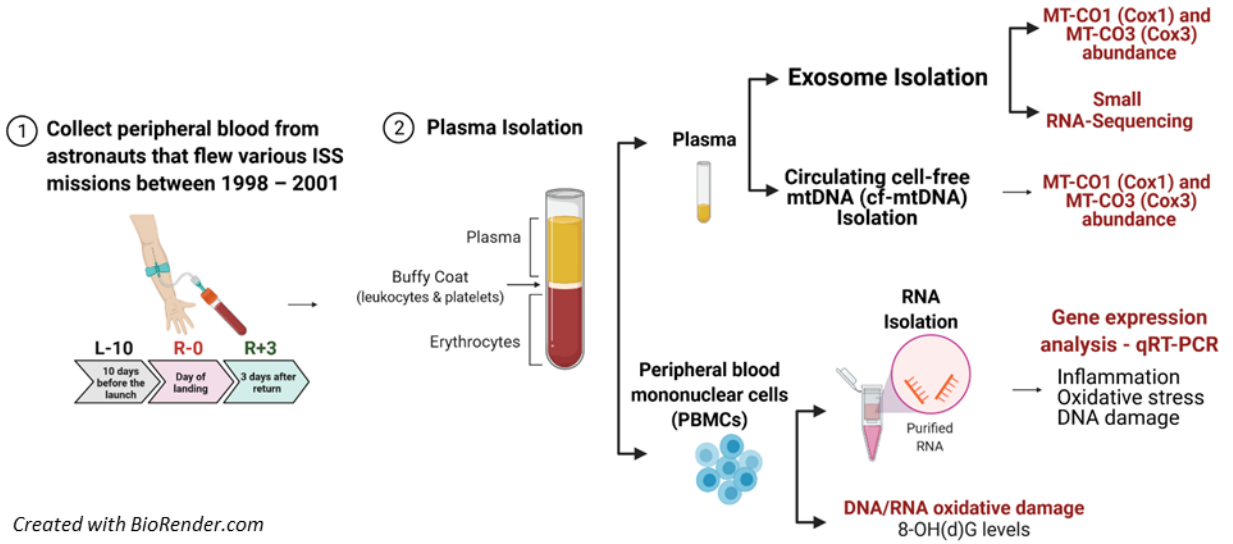
SUPPLEMENTAL MATERIAL

Data S1.

Supplemental Methods

Astronaut samples

We studied the levels of cf-mtDNA in the blood plasma of 14 astronauts who flew short (~5-13-day) ISS missions between 1998 and 2001. Information regarding de-identified blood samples, including Shuttle Space mission code, the approximate average age of the crew members, and time spent in space, along with experimental strategy, are depicted graphically and in the table below.



Sample ID #	Shuttle Mission Code	Approximate Age of The Crew	Time in Space
A1	STS99	45.2 ± 3.9	11d 5hr
A2	STS99	45.2 ± 3.1	11d 5hr
A3	STS106	42.0 ± 4.9	11d 19hr
A4	STS100	42.0 ± 4.1	11d 21hr
A5	STS102	44.8 ± 6.3	12d 21hr
A6	STS92	42.3 ± 4.0	12d 19hr
A7	STS92	42.3 ± 4.0	12d 19hr
A8	STS103	42.6 ± 5.5	7d 23hr
A9	STS100	42.0 ± 4.1	11d 21hr
A10	STS104	42.4 ± 3.1	12d 18hr
A11	STS104	42.4 ± 3.1	12d 18hr
A12	STS104	42.4 ± 3.1	12d 18hr
A13	STS93	44.2 ± 3.9	4d 22hr
A14	STS88	41.3 ± 4.1	11d 16hr

Blood was sampled at three different time points: 10 days before launch (L-10), the day of landing (R-0), and 3 days after return (R+3). Pre- and post-flight samples were stored at -80°C until use.

Nuclear and MT-DNA Measurements

cf-mtDNA was isolated using the DNeasy Blood and Tissue kit from Qiagen according to the manufacturer's protocol (Qiagen, USA). Mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) abundance was measured by real-time quantitative PCR (qPCR) using an Applied Biosystems 7900 Sequence Detection System (Applied Biosystems, USA). Primer pairs specific for mtDNA (MT-CO1 and MT-CO3) were designed to quantify mtDNA abundance, and human beta-globin primers were used for normalizing nDNA. Forward and reverse primer sequences are depicted below.

	Gene symbol	Species	Forward primer (5′-3′)	Reverse primer (5′-3′)
Mt-DNA abundance	MT-CO1	Human	GCCTCCGTAGACCTAACCATCTTC	GTAAGTTACAATATGGGAGATTATTCC
	MT-CO3	Human	ATGACCCACCAATCACATGC	ATCACATGGCTAGGCCGGAG
	GV1	Human	TTCACTAGCAACCTCAAACAG ACA	TGTCTCCACATGCCCAGTTTCT
	ACTB	Human	CTGGAACGGTGAAGGTGACA	AAGGGACTTCCTGTAACAATGCA

Thrombin plasma preparation for exosome precipitation

Exosomes were isolated from blood plasma samples of 3 astronauts (A9, A11, and A12) at L-10, R-0, R+3 using the ExoQuick Plasma prep and Exosome precipitation kit (Cat # EXOQ5TM, System Biosciences, CA, USA). In brief, 400 µl of plasma was mixed with thrombin and kept at room temperature (RT) for 5 minutes. Subsequently, samples were centrifuged at 10,000 rpm for 5 minutes. According to the manufacturer's protocol, the supernatant was collected into a new sterile microcentrifuge tube. Samples were then incubated with the exosome precipitation solution and refrigerated at 4°C for 30 minutes. After centrifugation at 1,500 x g for 30 minutes at 4°C, a beige-colored pellet was observed. Finally, the supernatant was aspirated, and the pellet was dissolved in 100 µl of sterile 1x PBS.

Exosomal DNA isolation

Exosomal DNA isolation was performed using the XCF Exosomal DNA isolation kit (Cat # XCF200S-1, System Biosciences, CA, USA). Isolated exosomes were dissolved in 1x PBS, and the final volume was adjusted to 500 µl. Next, the binding buffer was added to the PBS-dissolved

exosomes. Exosomes were centrifuged using a column provided by the manufacturer and further washed using the washing buffer. Exosomal DNA was eluted using the elution buffer, according to the manufacturer's protocol.

Exosome antibody array

The exosome antibody array was performed using the Exo-check exosome antibody arrays (Cat # EXORAY210A-8, System Biosciences, CA, USA). Briefly, isolated exosomes were quantified for protein using the BCA assay kit (Cat # 23225, Thermo scientific, Il, USA.). We used 50 µg of protein to incubate with the labeling reagent at RT for 30 minutes. Excess labeling reagent was removed according to the manufacturer's protocol. Labeled exosomes were blocked using a blocking buffer, and the membrane was exposed with exosomes facing up at 4º C overnight. The next day, the membrane was washed for 5 minutes at RT. The membrane was then incubated with the detection buffer at RT for 30 minutes. Subsequently, washing was done with wash buffer three times for five minutes at RT and developed using the chemiluminescence detection system (Clarity Western ECL substrate, cat # 170-5060S, Bio-Rad, USA).

Library preparation and small RNA sequencing

RNA quality was assessed using an Agilent TapeStation (Agilent, Palo Alto, CA, USA), and RNA concentration was quantified by Qubit 4.0 spectrophotometer. The library for small RNA sequencing was prepared using the Smarter smRNA-seq kit for Illumina (Takara Bio Inc., USA). The quantity and quality of amplified libraries were evaluated using Qubit (Invitrogen, Carlsbad, CA, USA) and Agilent TapeStation high sensitivity D1000 Screen Tape. Small RNA-seq libraries were sequenced using single-end 75 base pairs (PE75) sequencing chemistry on NextSeq 500 instruments following the manufacturer's protocols (Illumina).

Sequencing data analysis

Raw Fastq files were trimmed using cutadapt and built-in Illumina adapters. The quality of trimmed reads was assessed with FastQC, which is freely available at https://www.bioinformatics.babraham.ac.uk/projects/fastqc. Reads were aligned to human genome reference build GRCh38/hg38 with STAR aligner allowing for reads multi-mapping to different parts of the genome. GENCODE release 38 was used for gene and transcript annotations.

Raw counts were normalized by library size and transformed to log2 with edgeR package. The proportion of cf-mt-DNA vs. cf-nDNA gene expression was calculated as a ratio of total mt-DNA read counts to nDNA read counts. Differential expression of mt-DNA genes was assessed using limma.

Real-time quantitative reverse transcription PCR

We also isolated total RNA from peripheral blood mononuclear cells (PBMCs) of six astronauts pre-flight (L-10) and at two time points post-flight (R-0 and R+3). The real-time polymerase chain reaction was performed using SYBR green (Power up SYBR green master mix, cat # A25742, Applied Biosystem, USA) and QuantStudio™ 3 real-time PCR systems as recommended by the manufacturer. We measured the expression of genes encoding inflammation (*IL-6*, *IL-8*, *TNF-α, IL-1α, IL-β*), oxidative stress (*SOD1*, *SOD2*, *GPX1*, *NOX4*, *CAT1*, *NOXA1*, *SERPINE1*, *HMOX1*, *NOS2*, *NRF2*, *PRDX3*, *DUOX1*, *TMOD1*, *APOE*) and DNA damage markers (*OGG1*, *GADD153*, *GADD45a*, *PARP1*, and *DNAPK*). Please note, out of 14 astronauts, buffy coats were available only for individuals A7, A8, and A12. Forward and reverse primer sequences are depicted below.

Application	Gene symbol	Species	Forward primer (5′-3′)	Reverse primer (5′-3′)
RT-qPCR	APOE	Human	TGGGTCGCTTTTGGGATTACCT	AGGCCTTCAACTCCTTCATGGT
	CAT1	Human	CTTCTTGTTCAGGATGTGGTTTTCA	TACCTTTGCCTTGGAGTATTTGGTA
	DNAPK	Human	CAGGAGACCTTGTCCGCTG	AATACAAGCAAACCGAAATCTCTGG
	DUOX1	Human	AGAAATGCCAGCTGCCACTT	CCGCACATCTTCAACCAACACA
	GADD145A	Human	CGAAAGGATGGATAAGGTGGGG	GGATCAGGGTGAAGTGGATCTG
	GADD153	Human	CAGATGTGCTTTTCCAGACTGATC	TGATTCTTCCTCTTCATTTCCAGGA
	GAPDH	Human	CGACCACTTTGTCAAGCTCA	AGGGGAGATTCAGTGTGGTG
	GPX1	Human	AGGTACTACTTATCGAGAATGTGGC	TGAGGGAATTCAGAATCTCTTCGTT
	HMOX1	Human	GGTGATGGCCTCCCTGTACC	CTTGCGGTGCAGCTCTTCTG
	HSP60	Human	AGATGTAAAATTTGGTGCAGATGCC	CACACCATCTTTTGTTACTTTGGGA
	HSP70	Human	GTCCTAAGAATCGTTCAATTGGAGC	GCAACTGCACAATATCATATGCAAG
	HSP90	Human	CCCAGAGTGCTGAATACCCG	CTGTTTCCAGAGACAGAGTAGAGTG
	IL1A	Human	AAGAAGACAGTTCCTCCATTGATCA	CCTTGAAGGTAAGCTTGGATGTTTT
	IL1B	Human	ATGATGGCTTATTACAGTGGCAATG	ATCTTCCTCAGCTTGTCCATGG
	IL6	Human	ACAAGAGTAACATGTGTGAAAGCAG	ACTCTCAAATCTGTTCTGGAGGTAC
	IL8	Human	TTGCCAAGGAGTGCTAAAGAACTTA	AGCTCTCTTCCATCAGAAAGCTTTA
	NOXA1	Human	AACCATGATGCCAGGTCCCTAA	AGAGGAGCCTGTTTGCCAACTT
	NOS2	Human	ACACGTGCGTTACTCCACCA	GTCCCCTCTGATGCTGCCAT
	Nox4	Human	CTGTATAACCAAGGGCCAGAGTATC	TTATCCAACAATCTCCTGGTTCTCC

	NRF2	Human	CACTCACGTGCATGATGCCC	TGAGATGAGCCTCCAAGCGG
	OGG1	Human	ATCGTACTCTAGCCTCCACTCC	GTCTGAGTCAGTGTCCATACTTGAT
	PARP1	Human	GGATAAGCTCTATCGAGTCGAGTAC	CTTCCAGAAGCAGGAGAAGTGGTAC
	PRDX3	Human	TCAACGATCTCCCAGTGGGC	AGCAGCTGGACTTGGCTTGA
	SERPINE1	Human	ACATTCTGAGTGCCCAGCTCAT	ACATGTCGGTCATTCCCAGGTT
	SOD1	Human	CATCATCAATTTCGAGCAGAAGGAA	ATAGAGGATTAAAGTGAGGACCTGC
	SOD2	Human	CACATCAACGCGCAGATCATG	GATATGACCACCACCATTGAACTTC
	TMOD1	Human	GAGGAAGCCTTGGCAAATGCTT	AATTTGGTTCTTCGTCGGGCAC

DNA/RNA Oxidative Damage

DNA/RNA oxidative damage was measured in PBMCs isolated from 6 astronauts (L-10, R-0) and 5 astronauts (R+3) using the DNA/RNA Oxidative Damage (High Sensitivity) ELISA Kit (Cayman Chemical, USA). This competitive assay can be used to measure all three OxGua species: 8-hydroxyguanosine (8-OHG), 8-hydroxy-2’-deoxyguanosine (8-OHdG), and 8-hydroxyguanine. The antibody recognizes damaged nucleic acid species and binds to the goat polyclonal anti-mouse IgG previously attached to the well. The plate was washed to remove any unbound reagents, after which Ellman's Reagent was added to the well. The product of this enzymatic reaction has a distinct yellow color and absorbs strongly at 412 nm. The intensity of this color, determined spectrophotometrically, is inversely proportional to the amount of free 8-OHdG present in the well during the incubation.

Statistical analysis

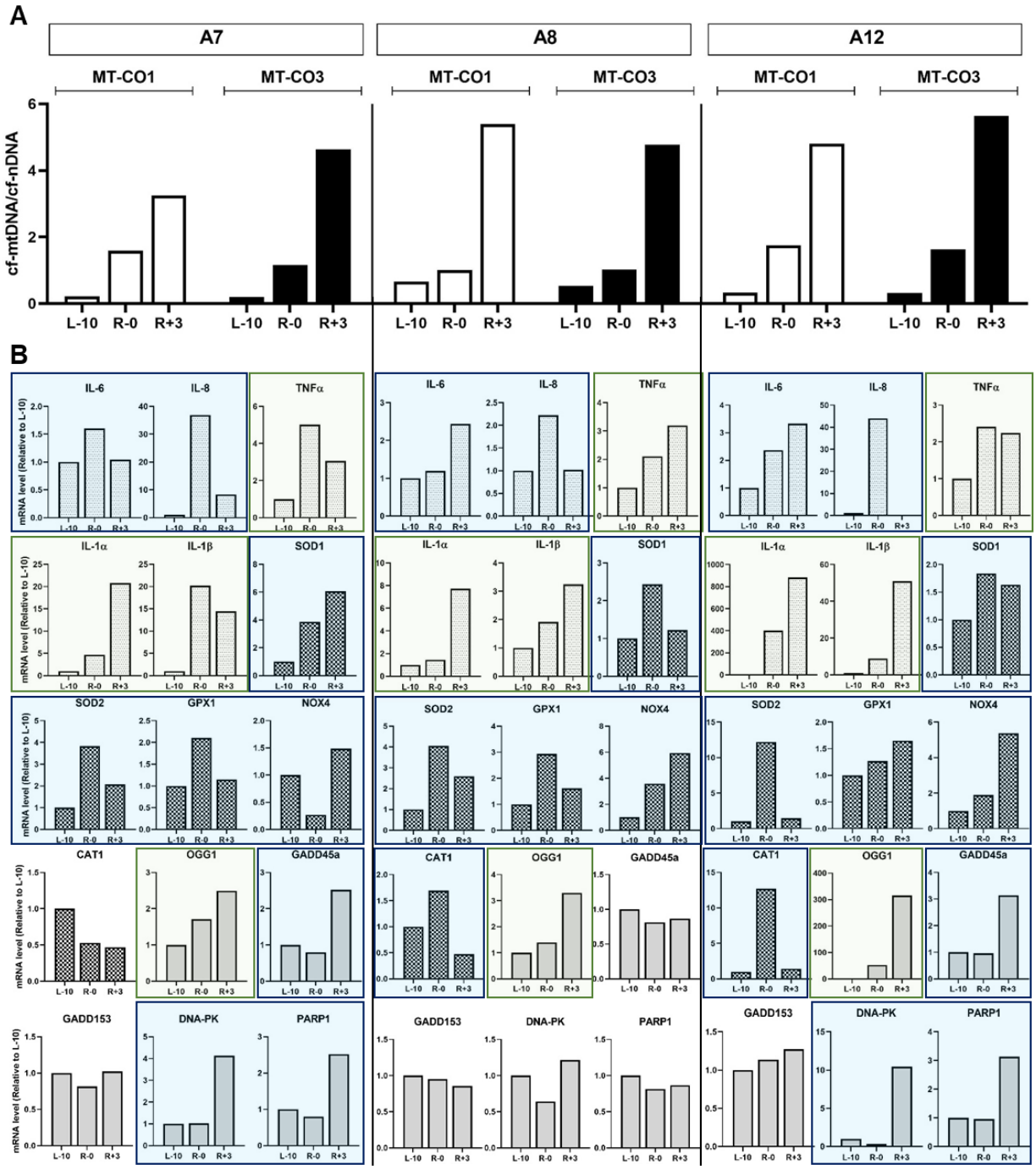
Results are presented as mean ± standard error of the mean (SEM) and were analyzed using a paired t-test for comparisons between means, or one-way ANOVA for repeated measures using the mixed-effects model followed by a Tukey post hoc test for multiple comparisons. Statistical analysis was performed using GraphPad Prism 6, version 6.07 (GraphPad Software, Inc., La Jolla, CA, USA). Differences were considered statistically significant at $p < 0.05$.

Table S1. List of differentially regulated genes.

Gene name	Fold Change								
	A9			A11			A12		
	L-10	R-0	R+3	L-10	R-0	R+3	L-10	R-0	R+3
MT-TF	11.91	11.72	11.18	12.58	9.99	11.24	11.27	10.78	10.45
MT-RNR1	14.77	14.59	14.76	14.59	14.94	14.63	14.41	14.82	14.9
MT-TV	14.24	13.87	14.23	14.42	10.92	13.48	14.22	13.98	12.01
MT-RNR2	19.57	19.61	19.45	19.42	17.79	17.77	19.46	19.7	18.02
MT-TL1	13.18	12.98	12.93	13.52	11.22	12.43	13	12.77	11.19
MT-ND1	12.99	13.23	13.28	12.07	13.47	13.64	12.76	13.6	13.34
MT-TI	9.91	9.89	8.84	9.47	8.12	8.96	10.03	9.79	8.53
MT-TQ	12.04	11.27	11.29	13.08	11.67	10.82	11.96	11.72	11.59
MT-TM	10.59	11.18	11.23	11.9	10.78	11.22	10.8	11.13	11.01
MT-ND2	14.8	15.23	14.89	14.89	15.33	15.21	16.32	15.57	15.37
MT-TW	11	10.66	11.09	11.49	11.13	12.03	10.44	11.05	11.45
MT-TA	11.96	10.21	11.19	11.75	10.27	11.55	10.85	11.22	9.74
MT-TN	10.66	10.21	10.49	11.28	10.59	14.27	10.63	10.76	10.36
MT-TC	11.44	11.01	11.22	11.12	10.08	11.1	11.18	10.97	10
MT-TY	10.65	10.14	10.19	11.4	9.43	9.49	11.37	10.93	9.74
MT-CO1	13.6	14.61	14.35	12.29	14.37	14.45	13.71	13.68	14.21
MT-TS1	13.62	13.04	12.98	14.42	12.25	13.02	14.45	14.11	12.39
MT-TD	12.12	11.42	12.3	12.36	9.45	11.56	12.06	11.58	10.11
MT-CO2	11.94	12.76	12.64	10.44	13.4	13.75	11.91	12.5	13.16
MT-TK	12.78	12.6	13.08	13.79	11.64	12.91	13.54	13.2	12.52
MT-ATP8	12.75	13.64	14.36	11.74	14.62	13.36	12.64	12.74	14.27
MT-ATP6	12.43	13.34	14.13	11.29	14.28	14.59	12.03	12.34	13.5
MT-CO3	13.67	14.31	13.83	12.93	15.04	13.63	13.64	13.89	14.97
MT-TG	15.62	15.23	15.01	15.93	11.37	12.41	15.77	15.42	12.21
MT-ND3	12.03	12.99	13	10.39	12.27	12.49	12.35	12.6	12.17
MT-TR	12.04	11.58	12.28	12.71	9.79	11.83	11.3	10.8	10.79
MT-ND4L	13.25	14	13.08	10.22	13.12	11.62	12.3	12.93	13.1
MT-ND4	13.83	14.68	14.54	12.85	14	14.18	13.71	15.36	13.94
MT-TH	11.46	11.17	12.18	12.16	10.74	12.38	11.64	11.49	10.98
MT-TS2	10.68	10.55	10.91	12.64	9.35	11.52	11.61	11.21	10.38
MT-TL2	14.7	13.76	13.66	15.15	10.37	12.21	14.09	13.94	11.5
MT-ND5	14.56	15.61	15.55	13.03	15.18	15.13	15.47	14.81	15.18
MT-ND6	11.94	13.12	13.08	10.28	12.98	12.83	12.04	15.26	12.67
MT-TE	11.37	11.48	11.15	12.56	10.48	12	12.06	11.91	11.08
MT-CYB	13.31	14.05	14.01	11.87	13.62	13.59	13.42	13.42	13.5
MT-TT	11.8	12.06	12.17	12.13	10.21	11.33	12.24	11.8	10.39
MT-TP	11.96	11.72	11.72	12.34	10.24	11.71	12.24	12.14	11.27

Gene expression of mt-DNA-encoded genes between control (L-10) and post-spaceflight (R0, R+3). Data are shown as fold change.

Figure S1. Comparison of cf-mtDNA and transcript levels of stress markers in PBMCs from three astronauts.



(A) Levels of cf-mtDNA at R0 and R+3 days in A7, A8, and A12 individuals. (B) Transcript levels of inflammatory markers (IL-1α, IL-1β, TNFα), and DNA damage markers (OGG1) follow similar trends to cf-mtDNA in 3 individual astronauts (light green background), whereas IL-6, IL8, SOD1, SOD2, GPX1, NOX4, GADD45, CAT1, DNA-PK, and PARP1 in at least one of the post-flight time points (light blue background).